RIBOZYMAL NUCLEIC ACID

Background of the invention

Field of the invention

This invention is in the field of recombinant DNA technology and relates to ribozymes.

Description of the related art

The art is currently exploring the many different ways of attacking the human immunodeficiency viruses (HIV), represented by HIV-1 and HIV-2 or the cellular mechanisms involved in infection by HIV and its progress *in vivo*. It has recently been shown that (HIV) and simian immunodeficiency virus (SIV) enter target cells by forming a complex between the viral envelope protein and two cell-surface membrane receptors: CD4 and a transmembrane chemokine receptor. Isolates of HIV that differ in cellular tropism use different subsets of chemokine receptors as entry cofactors: macrophage-tropic HIVs primarily use CCR5, whereas dual-tropic isolates use both CCR5 and CXCR4 (also called "Fusin") receptors; CXCR4 is also used by T-tropic viruses.

The role of CCR5 has been reviewed by S. O'Brien and M. Dean, Scientific American, 28-35 (September 1997). That review suggests several ways in which this stage of the HIV infection or maintenance might be blocked. These include:

- obstructing the binding site on CCR5 for HIV by use of chemokine derivative which competes with the natural chemokines or by use of synthetic antibodies,
- vaccination with fragments of CCR5,
- new genes whose products would prevent CCR5 from being made or would stop CCR5 from serving as a docking site for HIV.

PCT Application Publication No. WO 97/45543 proposes to inhibit the action or production of CCR5 receptors in a variety of ways including therapy with antibodies; making variants of CCR5 to use as decoys, thus interfering with the fusion of cells; by way of agents which bind to CCR5 and antisense oligomers and ribozymes to prevent expression of the DNA coding for CCR5.

PCT Application Publication No. WO 97/44055 similarly proposes many ways of inhibiting the action or production of CCR5 receptors, broadening the concept to extend to other chemokine receptors including CXCR4.

In Antiviral Agents Bulletin *10* (No. 9), 261-262 (September 1997), an article "Trojan Horse Virus' Controls HIV *in vitro*", it is reported that vesicular stomatitis virus (VSV) modified to express CD4 and CXCR4 can selectively target and kill HIV cells *in vitro*. This approach is described as problematical but "does highlight the potential to use HIV-mimicking attenuated viruses or liposomes with surface cellular HIV receptors for targeted delivery of HIV protease inhibitors, other antiretroviral drugs, antisense agents, gene therapies, ribozymes or other agents to HIV-infected cells."

Summary of the invention

It has now been found that mRNA coding for the CCR5 and CXCR4 proteins can be cleaved by hammerhead ribozymes so effectively as to block production of these proteins.

In one aspect, the invention provides a vector system comprising at least one DNA vector, the vector or vectors containing a target-cleaving hammerhead ribozymal DNA sequence under control of a promoter effective in human cells and which, upon transcription to RNA will cleave the mRNA transcribed from a target gene encoding the CCR5 or CXCR4 protein.

The linkage of the ribozymal DNA sequence to the promoter can be direct, and need employ only a single vector. However, there are advantages in an indirect linkage which amplifies the effect of the promoter. Such an indirect linkage will normally require two or more vectors. Thus, the invention includes a vector system comprising at least two DNA vectors, wherein a first vector contains a first promoter effective in human cells, operably linked to a gene which is expressible to produce an activator protein capable of acting on a second promoter, and a second vector contains the second promoter operably linked to the target-cleaving hammerhead ribozymal DNA sequence referred to above. The ribozymal DNA sequence can comprise a single sequence for cleaving the CCR5 or CXCR4 RNA or sequences for cleaving both CCR5 and CXCR4 RNA. The term "vector system" as used herein is generic terminology encompassing a single vector or a kit or composition or two or more vectors.

Strictly, a ribozyme is an RNA molecule which cleaves an RNA target. Some of the literature is using the term to describe DNA molecules which are transcribed to RNA, thus generating the ribozyme proper. In this specification, the term "ribozymal DNA" means DNA transcribable to the ribozyme proper.

The invention includes liposomes containing a DNA vector system as defined above and pharmaceutical compositions comprising the liposomes.

Still further, the invention includes the vector, composition and liposomes, as described above, for use in treating diseases associated with infections, especially HIV and AIDS. It includes also use of the nucleic acid, vector, or liposomes in the preparation of a medical formulation for such a purpose. Further, where patent law permits (e.g. Australia, USA) it includes a method of treating a patient suffering from a HIV-infection, which comprises administering to the patient the vector, composition or liposomes in an effective dose or as part of an effective dose when administered in conjunction with another treatment.

Further, the invention includes ribozymal DNA, both *per se* and as a ribozymal DNA sequence contained within a vector, the ribozymal DNA further comprising, downstream of the target-cleaving ribozymal sequence, a 3'-autocatalytic hammerhead ribozymal DNA sequence, so that when the ribozymal DNA is transcribed to RNA it has a form representable as a double hammerhead, having first and second stems of a target-cleaving ribozyme which targets CCR5 or CXCR4 mRNA and first and second stems of 3'-autocatalytic ribozyme, together with a common, third stem joining the two hammerheads. This third stem is preferably of at least 4 bases near the 3' end of the CCR5 or CXCR4 ribozyme sequence, capable of base-pairing with a complementary sequence of at least four bases near the 3' end of the autocatalytic ribozyme sequence, so as to form, when base-paired, the said common stem joining the hammerheads of the target-cleaving and 3'–autocatalytic ribozymes.

The verb "to comprise", whenever used herein in any grammatical form, means to consist of or include.

Additional Prior Art

AIDS Weekly Plus 19 May 1997, page 31, contains a report of an Abstract by R. Tritz et al. submitted to the "Keystone Symposia on Molecular and Cellular Biology entitled "Discovery and Development of Novel Therapeutic Agents for the 21st

Century", March 16-21, 1997, Tamarron, Colorado, USA. This abstract proposes that CCR5 is a suitable target for ribozyme gene therapy and reports that a number of hairpin ribozymes that target CCR5 RNA have been made.

Brief Description of the Drawings

Fig. 1 is a schematic drawing of a target-cleaving ribozyme sequence of the invention for CCR5;

Figs. 2 and 11 are schematic drawings of target-cleaving ribozymal DNA sequences linked to a 3'-autocatalytic sequence to provide a double hammerhead ribozymal DNA for targetting CCR5 and CXCR4 mRNA, respectively;

Figs. 3 and 12 show the DNA sequences of cassettes comprising the ribozymal DNA of Figs. 2 and 11, driven by a T7 promoter;

Figs. 4a and 4b are schematic drawings of target-cleaving ribozyme sequences used in this invention, in relation to CCR5 and CXCR4 mRNA targets;

Figs. 5a and 5b are photographs of agarose gels with ethidium bromide, containing human CCR5 mRNA and incubated with a ribozyme corresponding to the ribozymal DNA sequence provided by a vector system of the invention; these photographs shows how the CCR5 RNA has been cleaved after 1 hour of incubation (Fig. 5a) and after 3 hours of incubation (Fig. 5b);

Figs. 6a and 6b, 15a and 15b and 16a and 16b are plots of cell number (y-axis) against fluorescent intensity (x-axis) of cells untransfected (6a, 15a, 16b) and transfected (6b, 15b, 16b) with a liposome preparation containing a plasmid expressing a reporter gene encoding β-galactosidase (6b) or a plasmid vector system of the invention containing a ribozyme targeting CCR5 mRNA (15b) or CXCR4 mRNA (16b): the fluorescent intensity indicates the level of β-galactosidase (6a, 6b), CCR5 protein expression (15a, 15b) and CXCR4 protein expression (16a, 16b);

Fig. 7 is a map of plasmid pcDNA3 used in cloning CCR5 cDNA;

Fig. 8 is a schematic diagram of a plasmid pCS2 + NLS used for cloning of T7 polymerase DNA;

Fig. 9 shows diagrammatically some detail of the operations required for cloning of T7 polymerase in plasmid pCS2 + NLS of Fig. 8;

Fig. 10 is a schematic diagram of a plasmid pTM1 containing an internal ribosomal entry site (IRES) from the 5'-untranslated region (UTR) of encephalomyocarditis virus (EMCV);

Figs. 13a, 13b and 14 are photographs of agarose gels containing fragments of RNA transcribed from human peripheral mononuclear cells which have been transfected with ribozymes of the invention;

Fig. 17 is a schematic diagram of a 2-plasmid vector system of the invention, the first comprising a CMV-promoter driving a transcription of RNA from a T7 polymerase gene and the second comprising T7 promoter driving transcription of RNA from three ribozymal DNAs in tandem; and

Fig. 18 is a schematic diagram showing a 3-plasmid vector system of the invention, the first plasmid comprising a CMV promoter driving transcription of mRNA from a T7 polymerase gene, the second plasmid comprising a T7 promoter driving transcription of mRNA from a T7 polymerase gene and the third plasmid comprising a T7 promoter driving transcription of RNA from a ribozymal DNA which targets CCR5 or CXCR4 RNA or both.

Description of the Preferred Embodiments

The ribozymes in this invention are of the hammerhead type. Ribozymes have catalytic sequences which cleave the RNA at the desired target site. The catalytic sequences of hammerhead ribozymes are usually of the form (5' to 3')(1) cuganga ... and (2) ... gaa, where n is any nucleotide. In the present invention n is preferably u. They are separated by a stabilising structure, which is preferably a stem loop. The ribozymal DNA in the invention can have this form (substituting thymine for uracil).

The most preferred target sequences, for the purposes of the present invention, are

CCR5: 5' caaguccaaucua 3' (SEQ ID NO : 1)

CXCR4: 5' acaacgucagugag 3' (SEQ ID NO : 2)

The underlined portion is the essential sequence of three bases required by the hammerhead ribozyme used in the present invention.

Immediately upstream and downstream of the catalytic sequences lie target-binding (i.e. target-recognition) sequences. The target is RNA, and the ribozyme which

is RNA, is complementary to the target RNA, (disregarding the additional c nucleotide present in the target, as explained below).

The sequences involved in the preferred target and in the preferred ribozyme binding thereto may therefore be summarised as follows:

CCR5

(a) 5'	Caagu		c*	caaucua	3' (target RNA)
(b) 3'	guuca	-cat.seq.-s.l.	-cat seq.	-guuagau	5' (rz RNA)
(c) 5'	caagt	-cat.seq.-s.l.	-cat seq.	-caatcta	3' (rz DNA; strand 1)
(d) 3'	gttca	-cat.seq.-s.l	-cat seq.	-gttagat	5' (rz DNA; strand 2)

CXCR4

(a) 5'	acaacgu		c*	agugag	3' (target RNA)
(b) 3'	uguugca	-cat.seq.-s.l.	-cat.seq.	-ucacuc	5' (rz RNA)
(c) 5'	acaacgt	-cat.seq.-s.l.	-cat.seq.	-agtgag	3' (rz DNA; strand 1)
(d) 3'	tgttgca	-cat.seq.-s.l.	-cat.seq.	-tcactc	5' (rz DNA; strand 2)

(* = cleaved nucleotide, cat.seq. = catalytic site; s.l. = stem loop)

For the ribozyme targeting the CCR5 mRNA, 5' uagauug 3' is the first target-recognition sequence and 5' acuug 3' is the second target-recognition sequence. The cleavage site in the target is guc*, the asterisked c nucleotide being the cleavage site and therefore having no counterpart in the ribozyme. Sequences (a) and (b) for CCR5 are shown in Fig. 4 of the drawings. For the ribozyme targeting the CXCR4 mRNA 5' cucacu 3' and 5' acguugu 3' are the first and second target-recognition sequences. The cleavage site in the target is again guc*. A preferred sub-genus of ribozyme for use in the invention is those which have these target recognition sequences.

In the following description, the structure of hammerhead ribozymes is discussed in RNA terms, but it will be understood that the ribozymal DNA, from which they are transcribed, corresponds, substituting thymine for uracil. It will also be appreciated that the conformations of these ribozymes shown herein are those evident from base-pairing and other energetic considerations and that the invention is in no way

limited by these drawings, i.e. that the invention includes other conformations of the same molecules.

Hammerhead ribozymes are maintained by two stem loops, a first stem loop ("stem I") preceding the first target-recognition sequence and the second stem loop ("stem II") lying between the first and second target-recognition sequences. These two stem loops may have any desired form, but typically comprise 3 to 5 complementary base pairs forming the stem and 4 or 5 bases in the loop. Figure 1, relating to a ribozyme which targets the CCR5 mRNA, illustrates 4 base pairs in the stem portion proper and 5 bases in the loop portion. Figure 11, relating to a ribozyme which targets the CXCR4 mRNA, illustrates 5 base pairs in stem I, 4 in stem II and 4 base pairs in each loop portion.

Following the second catalytic sequence is a third stem, which consists of or includes the second target-recognition sequence. In one embodiment of the invention, stem III has a special sequence of guc at the 3' end which can be added in part or in full, if not naturally present. Where, as here, g is the natural ending, uc is added as an over-hang, see Fig. 1, so that the last few bases thereof pair with the bases of the second catalytic sequence. In Figure 1, this is achieved by a-u and g-c base pairs. This type of construction is suitable when the ribozyme does not contain a 3'-autocatalytic sequence but can also be used when it does.

More preferably, the ribozyme contains a 3'-autocatalytic sequence. Such sequences are known *per se*, especially from PCT Patent Application Publication N° WO 97/17433 (Medical University of South Carolina). The 3'-autocatalytic sequence is preferably designed so that a sequence near the 3' end of the target-cleaving ribozyme is base-paired with a downstream part of the autocatalytic sequence at or close to the 3-end thereof. These preferred constructs have at least 4 base pairs in stem III, i.e. 4 from the target-cleaving ribozyme which are base-paired with 4 from the autocatalytic ribozyme. They may have as many as 10 of these base pairs. Typically the over-hang of non-base-paired nucleotides extending beyond stem III is only 1 or 2 at the 3'-end of the target cleaving ribozyme sequence and 0 to 5 at the 3'-end of the autocatalytic sequence. Such constructions are exemplified for CCR5 in Figure 2 and for CXCR4 in Figure 11. Here, the 3'-autocatalytic (= self-cleaving) sequence is in the form of a hammerhead ribozyme comprising a 3'-cleavage site (guc), a first stem loop ("scas

Stem I"), a second stem loop ("scas Stem II") and a third stem ("scas Stem III"), the third stem being base - paired with Stem III of the target-cleaving ribozyme. Cleavage occurs after the c of the guc 3'-cleavage site. The catalytic sequence (cugauga) between scas stem I and scas stem II is one which assists in stabilising the hammerhead structure and is also used in WO 97/17433. Between Stems II and III there is provided a gaa catalytic site.

The ribozymes used in the present invention may contain a 5'-autocatalytic sequence, for example as described in WO 97/17433. In WO 97/17433 a double ribozyme is provided containing a centrally located *Bgl*II cloning site agatct into which any desired target-recognition and catalytic sequences can be inserted. In WO 97/17433, the insert is of 42 bases. These consist of first target-recognition sequence (8 bases), catalytic and structure-stabilising sequences (23 bases) and a second target-recognition sequence (11 bases, the last two of which are the ag of a *Bgl*II site). With minor modifications, the same construction could be adapted to the present invention, e.g. substituting the DNA equivalent of 36 bases, 13-48 of Figure 1, for the 23 bases of WO 97/17433, adding nucleotides necessary for cloning into a *Bgl*II site at the 3'-end thereof. In this construction Stem I would be dispensed with and replaced by the 5'-sequence of WO 97/17433 including the 5'-autocatalytic site. However, in WO 97/17433 the portion of sequence between the catalytic sites is not in a tight stem loop form and so appears less structure-stabilising.

The construct in the present invention is one or more vectors containing ribozymal DNA. To be effective in eukaryotic, especially human cells, the ribozymal DNA should be directly or indirectly controlled by a eukaryotic cell promoter. One especially such suitable promoter is the cytomegalovirus promoter. It is also preferable to provide other sources of RNA polymerase for transcription of the RNA, rather than rely wholly on endogenous polymerase in the cells in the body which are to be transfected. Preferably the polymerase is one which catalyses the action of the promoter, typically by acting on it *in trans*. That is to say, a first DNA molecule or length of DNA is expressed to produce a polymerase which acts on the promoter, contained in another DNA molecule or distinct length of DNA, which promotes transcription of the ribozymal DNA. Conveniently, DNA coding for the polymerase is provided within the vector, downstream of the ribozymal DNA. The polymerase

produced by the DNA will then act *in cis*, since it will "feed back" to stimulate its own the promoter. See e.g. Figure 17, plasmid 2 or the autopolymerase vector in Figure 18.

Referring to Figure 17, the plasmid 2 comprises a ribozymal DNA "rz1" which cleaves CCR5 or CXCR4 RNA or both. However, it has long been thought desirable to attack the HIV at more than one point in its cycle of infection, growth and replication. Thus, the same vector could contain one or more other kinds of ribozymal DNA which will target other RNA produced by HIV or required to make a protein on which HIV depends for its growth or replication. Thus, Figure 17 illustrates three kinds of ribozymal DNA as rz1, rz2 and rz3. Any one of these may be against CCR5 or CXCR4 RNA or both, while the other could be absent or could target another RNA produced by HIV or by a chemokine receptor. Especially preferred are ribozyme sequences targeting the mRNA of chemokine receptors CCR2b or CCR3. The RNA/DNA sequence of CXCR4 is disclosed in B. Federsppiel *et al.*, Genomics *16*, 707-712 (1993). The RNA/DNA sequences of CCR2b and CCR3 are also known, enabling ribozymes targetting these chemokine receptors to be developed, preferably analogously to those for CCR5 and CXCR4. For CCR2b, which was previously called human monocyte chemoattractant protein 1 receptor (MCP-1RB) see GenBank, Accession No. UO3905 and I.F. Charo *et al.*, Proc. Natl. Acad. Sci. USA *91*, 2752-2756 (1994) and for CCR3 see GenBank, Accession No. U28694 and C. Combadiere *et al.*, J. Biol. Chem. *270*, 16491-16494 and 30235 (1995) and *ibid.*, *271*, 11034 (1996). The role of CCR2b and CCR3 in HIV infections is described by B.J. Doranz, Cell *85*, 1149-1158 (1996) and by R.I. Connor. J. Exp. Med. *185*, 621-628 (1997).

Another target is a sequence at the 3'-end of the HIV viral mRNA (a 5' leader sequence in DNA terms). See PCT Application Publication N° WO 97/07667 (University of California), which describes a hairpin ribozyme and identifies the target.

In order to "kick start" the promoter it is desirable to provide a separate source of the polymerase, either as the enzyme itself or, more preferably in the form of another vector, which is also preferably a plasmid, dedicated for this purpose. See Figure 17, plasmid 1 and Fig. 18.

Referring to Fig. 18, a first plasmid contains the CMV promoter driving transcription of the T7 polymerase gene, a second plasmid contains the T7 promoter and a translational enhancer (exemplified as an IRES and illustrated as from EMC virus) for

the production of T7 polymerase and a third plasmid in which a T7 promoter, activated by the polymerase produced by the first two plasmids, drives transcription of the CCR5 and/or CXCR4 ribozymes. Other translational enhancers could be used in place of that shown.

Other targets are the LTR (long terminal repeat) and *tat* gene regions of HIV. Hammerhead ribozymes for this purpose are described in PCT Publication WO 95/04818, which also contains a bibliography of other HIV genes previously targeted, including the *gag* gene [Chang *et al.*, Clinical Biotechnology *2*, 23 (1990) and N. Sarver *et al.*, Science *247*, 1222-1225 (1990) and the *vif* gene [E. U. Lorentzen *et al.*, Virus Genes *5*, 17-23 (1991)].

In order to increase efficiency, the promoter can be inserted in front of each ribozyme sequence as well as the polymerase sequence, as shown in Fig. 17. However, this arrangement can be varied by using a single promoter, altering the order of the genes. Also, by using several vectors in place of plasmid 2, expression of the ribozymes can be varied.

Methods of delivery that may be used include encapsulation in drug delivery vehicles, especially, liposomes, transduction by retroviral vectors, and conjugation with cholesterol.

Drug delivery vehicles are effective for both systematic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. Some examples of such specialized drug delivery vehicles are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

Liposomes are preferred. They are hollow spherical vesicles composed of lipids arranged in a similar fashion as the lipids of the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Liposomes can deliver the DNA to cells, so that the nucleic acid remains biologically active.

They can easily be prepared by mixing the DNA with a liposome-forming lipid such as a dialkyl or diacylglycerol or phosphatidinylcholine, as known in the art of liposome formation. See J. J. Rossi *et al.* AIDS Research and Human Retroviruses *8*, 183-189 (1992).

Liposome preparations useful in the invention comprise: (a) lipofectamine reagent (GIBCO BRL, Gaithersburg, MD USA) containing a polycationic lipid molar ratio, (b) the cationic lipid, DDAB and DOPE, in a 2:1 ratio, R. Philip, Mol. Cell. Biol. *14*, 2411-2418, (1994); and (c) DMRIE, optionally in combination with DOPE, e.g. in a 1:1 molar ratio (VICAL Corp. San Diego, CA, USA). Newer liposomes, for example the serum-resistant cationic lipid GS 2888, J. G. Lewis *et al.*, Proc. Natl. Acad. Sci. USA *93*, 3176 (1996) and liposomes containing a polylysine/DNA complex, S. Li and L. Huang, J. Liposome Research 7, 63-75 (1997), can also be used.

Nanoparticles and hydrogel carriers have been developed for chemotherapeutic agents and protein-based pharmaceuticals, and consequently, can be adapted for ribozyme delivery for the purposes of the present invention.

Another delivery method is *via* T-cells. Compatible T-cells, preferably the patient's own are infected with ribozymal DNA of the invention, for example by electroporation and the patient is then infused with these cells. Electroporation of T-lymphocytes with DNA is described in Example 6 of PCT Publication WO 96/22638 (Gene Shears Pty Ltd.) and this method can be applied in the present invention.

The compositions for pharmaceutical use will normally contain a magnesium salt, preferably as buffered magnesium chloride, this being required for the function of the ribozyme. They may also contain a carrier or diluent, which can include a suspending or emulsifying agent.

Vector systems of the invention, preferably in a liposome formulation, are preferably systemically administered, e.g. by an intravenous, subcutaneous, intraperitoneal, intranasal or intrathecal route. The dosage of ribozyme provided by the vector system will depend upon the disease indication and the route of administration but should be up to 200 mg/kg and usually at least 10 mg/kg of body weight/day. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

The following Examples illustrate the invention.

Examples

1. CCR5 DNA Cloning and Expression

Human CCR5 mRNA was isolated from the blood sample of a normal individual as follows. Human peripheral blood mononuclear cells (PBMC) were cultured as described in "Methods of Immunological Analysis", R. F. Masseyeff, W. H. Albert and N. A. Staines, pub. V.C.H. Verlag, Weinheim Germany (1993), Vol. 3 "Cells and Tissues", pp. 121-135 and isolated using gradient centrifugation. The cells were then stimulated with red kidney bean lectin (PHA-L, Sigma) at 1ug/ml for 24 hours. The stimulated cells were treated with 4M guanidinium thiocyanate and SDS solution and then subjected to acid-phenol separation and isopropanol precipitation procedures, all in accordance with the procedure described in "Molecular Cloning: A Laboratory Manual", ed. J. Sambrook, E. F. Fritsch and J. Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2nd ed. 1989. cDNA was then made with oligo dT (16-18 bases) and reverse transcriptase by a standard procedure. PCR was performed with the primers designed on the basis of the DNA sequence disclosed in M. Samson *et al.*, Biochemistry *35*, 3362-3367 (1996). Thus, the sequence of the forward primer was

5'-tgcacagggt ggaacaagat gg-3' (SEQ ID NO : 3)

and the sequence of the reverse primer is

5'-cacttgagtc cgtgtcacaa gc-3' (SEQ ID NO : 4)

Taq polymerase was used, which gave an a (adenosine) overhang ending to the 3'-end. The PCR product was then cloned into pcDNA3, a 5.4 Kb plasmid sold by Invitrogen UK Ltd, (see Figure 7) in a *Eco*RV restriction site. Then, t (thymidine) nucleotides were added to the 3'-ends of an *Eco*RV restriction site of the vector, so as to produce a "ta cloning" site in the vector. "ta cloning" is described by J. M. Clark, Nucleic Acids Research *16*, 9677-9686 (1988), in US Patent 5,487,993 and on the Worldwide Web at http://www.gene-labs.com/pro42.htm. The plasmid was then transfected into *E. coli* XL1-blue and screened. The CCR5 DNA insert was completely

sequenced and found to be identical to the published CCR5 DNA sequence. As shown in Fig. 7, pcDNA3 contains a T7 promoter before the multiple cloning site. CCR5 mRNA transcripts were produced by adding T7 polymerase in "Ribomax", solution as described by the "Protocols and Applications Guide" of Promega Ltd.

2. CCR5 Ribozymal DNA Cassette

A complete ribozymal DNA cassette was constructed having the nucleotide sequence SEQ ID NO:5, described more fully in Fig. 3:

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aatctagagg      atcctaatac      gactcactat
agggcgaaag      ccctagattg      ctgatgagcg
cgaaagcgcg      aaacttgtcc      tctacgaaag
tagagctgat      gagaccgaaa      ggtcgaaaca
agtgagctcg      aattctt         137
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It was made from a forward oligomer from positions 1 to 80 of the coding sequence and a reverse one from positions 137 to 56, using an oligonucleotide synthesizer. 25 bases at the 3'-ends of the oligomers were totally complementary to each other and the two strands were annealed. Their elongation to become a complete double strand was carried out with DNA polymerase on a PCR machine. The 137-long ds DNA cassette was cloned into pUC19 using *Xba*I and *Eco*RI sites (see Fig. 3) for the 5'-end and 3'-end respectively. Its sequence was confirmed by DNA sequencing. The cassette contains a T7 promoter, as the commercially available pUC19 does not contain this site.

Referring to Figures 3 and 1, the cassette comprises in order (5' to 3'):

Figure 3 Base Nos.	Figure 1 Base Nos.	Function
1-14		Vector sequence.
15-31		T7 promoter.
32-43	1-12	First structure-stabilising stem loop of the target-cleaving ribozymal DNA ("rzsccr5 stem I").
44-50	13-19	First target-recognition sequence (binding site).
51-57	20-26	First catalytic sequence of the target-cleaving ribozymal DNA.
58-69	27-38	Second structure-stabilising stem loop of the target-cleaving ribozymal DNA ("rzsccr5 stem II").
70-72	39-41	Second catalytic sequence of the target-cleaving ribozymal DNA (gaa), forming part of stem III ("rzsccr5 stem III")
73-79	42-48	Second target-recognition sequence, ending in g with uc overhang, forming the remainder of rzsccr5 stem III.
80-95		First structure-stabilising stem loop of autocatalytic ribozymal DNA ("scas stem I").
96-102		First scas catalytic site.
103-114		Second structure-stabilising stem loop of autocatalytic ribozymal DNA ("scas stem II").
115-123		Bases gaa forming the second scas catalytic site, followed by a 6-base sequence which base-pairs with 6 complementary bases of the second target-recognition sequence above, thus forming another stem ("scas stem III").
124-137		Vector sequence (includes g overhang as nt 124).

The ribozymal RNA was made by adding T7 polymerase in "Ribomax" solution, as described by Promega's "Protocols and Applications Guide" manual. "Ribomax" is a balanced salt solution containing the necessary magnesium ions for ribozymal activity. T7 polymerase triggers the T7 promoter to transcribe RNA from the DNA. Transcripts were isolated by using RNase-free DNase, followed by acid/phenol isolation of RNA, then ethanol precipitation. It was done as described in "Molecular Cloning - A Laboratory Manual", cited above.

When the RNA was run on an agarose gel, the ribozyme before cleavage (103 bases) was clearly separated from the ribozyme after cleavage (48 bases, Figure 1). RNA was shown by ethidium bromide contained in the gel (visualised under UV light).

3. Human CCR5 Target Cleavage

Human CCR5 DNA prepared as described in Section 1 above, cloned into pcDNA3, was transfected into *E.coli* XL1Blue ("Molecular Cloning - A Laboratory Manual", cited above) to produce anRNA transcript of CCR5.

Ribozymes transcribed from the plasmid described in Section 2 above were incubated with the CCR5 RNA transcript at a molar ratio of 1 mole ribozyme to 10 moles of CCR5 RNA transcript. Within 3 hours of incubation at 37°C, total cleavage of the CCR5 mRNA target was achieved. The RNA was run on 2% agarose gel containing ethidium bromide. Gel photographs taken under UV irradiation are presented in Figures 5a and 5b. Figure 5a, left-hand lane, shows the products after 1 hour of incubation, when the CCR5 mRNA was not completely cleaved and so appeared as a band of high molecular weight at the top. The right-hand lane consists of molecular weight markers. Also present in the left-hand lane were bands of lower molecular weight, attributable (in descending order of molecular weight) to a 3'-fragment of the CCR5 mRNA after cleavage, a 5'-fragment of the CCR5 mRNA after cleavage and the ribozymal RNA. Thus it is clear that the rzsccr5 ribozyme can self-cleave to produce an active ribozyme that cleaves CCR5 catalytically. Referring to Figure 5b, the right-hand lane shows the products after 3 hours of incubation, when the CCR5 mRNA has been completely cleaved. The band representing uncleaved mRNA in Figure 5a has disappeared and has been replaced by a band corresponding to the 3'-end of the cleaved product, at lower molecular weight. The fragment containing the 5'-end of the CCR5 mRNA is visible at even lower molecular weight than in Figure 5a. There is no visible band containing CCR5 ribozyme. This is because the gel was loaded with a sample ten times more dilute than that used in Figure 5a.

4. Transfection of Plasmids into PBMC

Plasmids were transfected into normal human peripheral blood mononuclear cells (PBMC) obtained from a blood bank. The cells were isolated, stimulated with PHA-L and IL-2 using the standard procedure of S. J. Martin *et al.*, J. Immunol. *152*, 330-342 (1994). Six days later the cells were treated by a procedure adapted from that

of Gibco Life Technologies Ltd., as follows. 12 µl of DMRIE-C (Life Technology Ltd.), a liposome-forming preparation, were mixed with 0.5 ml of OPTI-MEM I reduced serum medium. 20 µl or 200 µl of plasmids were added in 0.5 ml of OPTI-MEM I medium to the mixture and mixed well with it. The solution was then left at room temperature for 45 minutes before 0.2 ml of PBMC suspension containing 4 million cells was added. The cellular suspension was prepared with 1/4 culture supernatant and 3/4 RPMI, topping up with IL-2 to 10 U/ml. The final mixture was incubated at 37°C for 4-5 hours with 5% CO_2. Then, the cells were resuspended in normal culture medium (RPMI1640, IL-2 10 u/ml and 10% Foetal Calf Serum) by adding the mixture in aliquots to the medium. The viability at this point was more than 90% by Trypan Blue staining. The cells were then cultured in the normal way for analysis.

The above procedure was carried out initially using a 9.8 Kb plasmid, containing the murine Moloney Leukemia Virus promoter and the β-galactosidase gene, a reporter gene. On day 8 after transfection the β-galactosidase product was analyzed by FACS-FDG stain after the inhibition of the endogenous β-galactosidase (Reagents and Protocol, Molecular Probes, USA). As shown in Figures 6a and 6b, where cell number as a percentage is expressed on the vertical axis against fluorescent intensity on the horizontal axis, there was a clear shift of the fluorescein isothiocyanate (FITC) intensity, which indicates a successful transfection of plasmids to human PBMCs.

This procedure, with minor alterations, can be carried out using plasmids of the invention carrying the ribozymal DNA, as shown in Section 9.

5. CXCR4 Ribozymal DNA

Human CXCR4 ribozymal DNA was prepared analogously to human CCR5 ribozymal DNA as in Section 1 above. The CXCR4 DNA sequence is disclosed in B. Federsppiel *et al.*, Genomics *16*, 707-712 (1993). The sequence of the forward primer was

```
5'-gccaagcttc  tgcagtaata  cgactcacta
tagggccgaa     aggcccctca  ctctgatgag
cgcgaaagcg     cgaaacgttg  tcctctg-3
```

(SEQ ID NO:6)

and of the reverse primer was

```
5'-taattggatc  ctctagaaac  gttgtttcgg
tcctttcgga     cctcatcagc  tctgatttct
cagaggacaa     cgtttcgcgc  tttc-3'
```

(SEQ ID NO:7)

The ribozymal DNA cassette was constructed having the nucleotide sequence SEQ ID NO:8, described more fully in Fig. 12:

```
gccaagcttc  tgcagtaata  cgactcacta
tagggccgaa  aggcccctca  ctctgatgag
cgcgaaagcg  cgaaacgttg  tcctctgaga
aatcagagct  gatgaggtcc  gaaaggaccg
aaacaacgtt  tctagaggat  ccaatta      147
```

Referring to Figure 12, the cassette comprises in order (5' to 3'):

Figure 12 Base Nos.	Function
1-15	Vector sequence.
16-32	T7 promoter.
33-46	First structure-stabilising stem loop of the target-cleaving ribozymal DNA ("rzscxcr4 stem I").
7-52	First target-recognition sequence (binding site).
53-59	First catalytic site of the target-cleaving ribozymal DNA.
60-71	Second structure-stabilising stem loop of the target-cleaving ribozymal DNA ("rzscxcr4 stem II")
72-74	Second catalytic site of the target-cleaving ribozymal DNA gaa, forming part of stem III (rzscxcr4 stem III").
75-82	Second target-recognition sequence, ending in gu with c overhang, forming the remainder of "rzscxcr4 stem III".
83-98	First structure-stabilising stem loop of autocatalytic ribozymal DNA ("scas stem I")
99-105	First scas catalytic site.
106-119	Second structure-stabilising stem loop of autocatalytic ribozymal DNA ("scas stem II").
120-132	Bases gaa forming the scas catalytic site, followed by a 10-base sequence which base-pairs with 10 complementary bases of the second target-recognition sequence and catalytic site above, thus forming another stem ("scas stem III").
133-147	Vector sequence.

6. Engineering of the polymerase vector

Referring to Fig. 18, the polymerase vector comprises a promoter from cytomegalovirus (CMV) and the T7 polymerase gene. The complete T7 polymerase DNA sequence is available from Genbank/EMBL under Accession No. M.38308. In this Example, a modified T7 polymerase DNA was obtained and amplified by PCR on plasmid pT7AutoI [J. Dubendorff and F. Studier, J. Mol. Biol. *219*, 61-68 (1991)]. The primers used for the PCR incorporated the restriction sites *Eco*RI and *Nco*I at the 5' end of the forward primer; and *Bam*HI, at the 5' end of the reverse primer. (*Bam*HI was used later for the cloning of the autopolymerase vector.) The sequences of the primers were as follows with the overlapping T7 polymerase underlined.

Forward:

5' acgaattccatggacacgattaacatcg 3' (SEQ ID NO: 9)

*Eco*RI site = gaattc; *Nco*I site = ccatgg

Reverse:

5' atataaggatccttacgcgaacgcgaac 3' (SEQ ID NO: 10)

*Bam*HI site = ggatcc

The PCR was carried out using Vent polymerase (which provided a 'blunt end' in the PCR product). The *Nco*I site introduced by the forward primer is an extra cloning site and was produced by changing the second codon of the T7 polymerase, DNA from an Asn (aac) to an Asp (gac). This does not change the activity of T7 polymerase.

The T7 polymerase PCR product was cloned into a pCS2-NLS plasmid (R.A.W. Rupp *et al.* Genes and Development *8*, 1311-1323 (1994) and D.L. Turner & H. Weintraub *ibid.* 1434-1447], Fig. 8 is a map of the plasmid pCS2-NLS. The T7 polymerase DNA was introduced into an *Eco*RI site and a *Sna*B1 (blunt ended) site in the CS2, located shortly after the NLS in the clockwise direction. The NLS sequence in the pCS2 was unnecessary for the present purpose at this stage and it was deleted by cutting with restriction enzyme *Nco*I as the NLS sequence was now in between the two

*Nco*I sites. Then the plasmid was religated through the *Nco*I sites. Fig. 9 explains these operations. Thus the polymerase plasmid vector was completed as shown in Fig. 18. It contained a CMV promoter (already existing in the CS2 vector), which switched on the production of T7 polymerase. T7 polymerase is required for the ribozymal DNA vector and the autopolymerase vector, detailed below.

7. Engineering of the autopolymerase vector

The purpose of this vector was to provide a steady and adequate supply of T7 polymerase. The T7 promoter was used to switch on the production of T7 polymerase. This polymerase acted autocatalytically making more T7 promoter which made more T7 polymerase (Fig. 18).

mRNAs made by transfected vectors through non-mammalian promoters in mammalian cell cytoplasm are not usually recognised by the cells for translation into proteins. In order to trick the cell into translating the T7 polymerase mRNA transcribed by the promoter, an encephalomyocarditis (EMC) virus. UTR (untranslated region) sequence, (Moss *et al.*, Nature *348*, 91-92 (1990)) was added. This sequence serves as a translational enhancer, providing binding sites for ribosomes and was obtained by PCR-amplifying EMC UTR from the pTM1 vector, see B. Moss *et al.*, Nature *348*, 91-92 (1990). The primers used included an *Xba*I restriction site in the forward strand. No restriction site was introduced in the reverse primer, since the EMC sequence obtained from this vector contained several engineered restriction sites, such as *Bam*HI and *Nco*I. The primers were as follows, with the overlapping EMC sequences underlined:

Forward:

5' gctctagaccacaacggtttccctctag 3' (SEQ ID NO: 11)

*Xba*I site = tctaga,

Reverse:

5' cagcttcctttcgggctttgttagcagc 3' (SEQ ID NO: 12)

The EMC sequence was then cloned into pET11a (Novagen Ltd.) using *Xba*I and *Bam*HI sites. For a map, see e.g. the 1996/97 catalogue of R & D Systems Ltd.,

Abingdon, Oxfordshire, England. page 74. The EMC UTR sequence naturally contained a *Nco*I restriction site at its 3' end, in front of a *Bam*HI site. Thus, the T7 polymerase sequence, as described above in Section 6, which contained *Nco*I and *Bam*HI sites, was readily cloned into the plasmid downstream of the EMC UTR sequence, as described by X. Chen *et al.*, Nucleic Acids Research *22*, 2114-2120 (1994).

8. Engineering of the ribozyme vectors

The cassettes containing the complete ribozymal DNA and T7 promoter were cloned into the well known plasmid pUC19. For one set of experiments, human CCR5 ribozymal DNA was cloned into the plasmid using *Xba*I and *Eco*RI sites. For another set of experiments, the CXCR4 cassette was cloned into same plasmid just in front of the CCR5 cassette. This was done using *Pst*I and *Xba*I restriction enzymes. The CXCR4 cassette has a *Pst*I site near its 5'-end and an *Xba*I site near its 3'-end (Fig. 12). Restriction with *Xba*I carries the 3'-end of the CXCR4 cassette to be ligated directly onto the *Xba*I site near the 5'-end of the CCR5 cassette (Fig. 3). The resultant plasmids conform to the "ribozyme vector" shown in Fig. 18.

9. Expression of the vectors in human peripheral blood mononuclear cells, showing powerful inhibition of CCR5 and CXCR4 RNA

Human peripheral blood mononuclear cells (PBMC) were isolated and cultured as described in Section 1. Various of the three vectors, i.e. polymerase, autopolymerase and ribozymal DNA, as shown in Fig. 18, were added in equal proportions for transfection. Three experiments were carried out (1) in which all three vectors were added, (2) in which only the polymerase vector and ribozymal DNA vector were added and (3) in which only the polymerase vector was added.

Transfection was carried out as described by Gibco Ltd. which supplied DMRIE-C reagent. 8µl of DMRIE-C was mixed with 4µg in 1ml of Optic-MEM before $2x10^6$ cells were added for transfection, as described by Gibco's instructions. Cells were then stimulated with PHA-L (Sigma) at 1µg/ml and IL-2 10U/ml for 48 hours (final volume, 3ml) before they were harvested for analysis. In a first set of experiments, the ribozymal DNA plasmid containing only CCR5 ribozymal DNA was used. In a second set of experiments the PBMC were transfected with a plasmid containing both CXCR4 and CCR5 ribozymal DNA in a single plasmid (Figure 18, Section 8 above).

RNA was isolated from the transfected cells as described in Section 1 and PCR was carried out to identify cellular CCR5 or CXCR4 mRNA using the above-described CCR5 or CXCR4 primer. Also, as the cells naturally express actin, the amount of mRNA of actin provides a quantitative control. Such a control confirms that the inhibition of CCR5 and/or CXCR4 is not due to the general degradation of RNA; otherwise the actin RNA would have degraded too. It also confirms that the ribozyme action is specific, in that it does not cleave actin RNA. This control, using actin, is widely applied in molecular biology.

The results are shown in Figures 13a, 13b and 14 which are stained agarose gel photographs of the relevant PCR products. Figs. 13a and 13b relate, respectively, to the first and second sets of experiments. Fig. 13a showing the action of the ribozyme against CCR5 RNA and 13b showing the action against CXCR4 RNA. The arrangement of Figures 13a and 13b is the same, and is as follows, numbering the lanes 1-8 from left to right:

mRNA type	**Lane No.**	**Polymerase vector present in vector system**	**Autopolymerase vector present in vector system**	**Ribozymal DNA vector present in vector system**
CCR5 or CXCR4	1	Yes	Yes	Yes
"	2	Yes	No	Yes
"	3	Yes	No	No
"	4	[Molecular weight markers]		
Actin	5	Yes	Yes	Yes
"	6	Yes	No	Yes
"	7	Yes	No	No
"	8	[Molecular weight markers]		

Lane 1 shows that CCR5 and mRNA was not deletable from the PBMC transfected with all three vectors, i.e. the polymerase, the autopolymerase and the ribozymal DNA vectors, thus indicating complete inhibition of CCR5 mRNA. Lane 2 contains a weak band of CCR5 mRNA, showing that without the autopolymerase vector, the inhibition of CCR5 and CXCR4 mRNA was incomplete. Lane 3 contains a bright band of the CCR5 or CXCR4 RNA, showing that without the ribozyme vector

there is no inhibition of CCR5 (Fig. 13a) or CXCR4 (Fig. 13b) mRNA. The inhibition of the CCR5 or CXCR4 RNA was specific, as the controls in Lanes 5-7 show clearly a bright band of actin mRNA from the same transfected cells as in Lanes 1-3. Thus, specific and powerful inhibition of CCR5 and CXCR4 has been achieved by the present invention. Although the powerful inhibition required the autopolymerase vector, those skilled in the art will be able to devise alternative ways of boosting ribozymal production to equivalent or greater levels. This can be done by increasing the concentration of the ribozymes using for instance, multicopies of ribozymal DNA sequence in the vector or vectors and/or by increasing the amount and/or efficiency of promoters.

All of the T7-related work described herein followed the protocols and recommendations by Novagen Ltd, including pLysS gel purification. The rest followed the protocols in "Molecular Cloning – A Laboratory Manual", 2nd ed. 1989 ed. Sambrook, Fritzsch and Maniatis.

As mentioned earlier, the ribozymal DNA vector containing CXCR4 ribozymal DNA also contains CCR5 ribozymal DNA. The mRNA level of CCR5 from PBMCs treated with this vector was monitored and the result is shown in Fig. 14. Referring to Fig. 14, lanes are numbered right to left. Lane 1 is molecular weight markers, Lane 2 containing a large bright band, is PBMCs treated with a control vector CS2 containing no ribozyme. Lane 3 shows PBMCs treated with polymerase and ribozymal DNA vectors only, Lane 4 with polymerase, autopolymerase and ribozymal DNA vectors. A complete cleavage is seen, as indicated by the disappearance of the CCR5 band. This has also provided clear evidence that the self-cleaving ribozyme is capable of producing multiple ribozymes from the same vector.

10. Effects on PBMC cellular expression of CCR5 and CXCR4 protein molecules after treatment with ribozymes

Following the transfection as described in Section 9, human PBMCs from individual donors (un-pooled) were stimulated with PHA-L and IL-2 as described in Section 4 or stimulated with a monoclonal antibody to CD3 called UCHT-1 (available from Dept. of Immunology, University College Hospital, London, UK) with IL-2 at 10 U/ml as described by Masseyeff et al. in "Methods of Immunological Analysis" (pub. VCH Verlag, Weinheim Germany (1993)). The cells were stained at days 3, 5, 7, 10,

12 with monoclonal antibodies to CCR5 or CXCR4 or control antibodies which were directly conjugated with fluorescent materials, commercially available from PharMingen Ltd. The staining procedure followed the instructions from the same company. The data were analysed using a Fluorescent Activated Cell Sorter (Becton Dickinson Ltd) as in Section 4. The results are summarised below as well as in Figs. 16a, 16b (CCR5) and 17a, 17b (CXCR4). Those Figures are similar to Figs. 6a and 6b referred to in Section 4. Here they show a clear shift of cell population to the left, indicating decrease in the expression of CCR5 or CXCR4.

The best inhibition was seen on different days in each different individual. On average, CCR5 ribozyme inhibited 78% of the expression of CCR5. CXCR4 ribozyme (which also contained CCR5 ribozyme) inhibited CXCR4 expression by 69% and CCR5 expression by 79%.

11. Inhibition of HIV infection in human peripheral blood mononuclear cells (PBMCs) treated with the ribozymes

Following the transfection as described in Section 9, the cells were stimulated with PHA-L and IL-2 as described in Section 4. These cells were pelleted on day 5 before the incubation with 100 µl of a HIV strain called LAI, available from MRC (Medical Research Council, UK). It contained 3000pg of p24 HIV surface antigen protein, as determined by sandwich ELISA supplied by Coulter Ltd. (This is the same assay used clinically to determine the amount of virus in patients' blood.) The mixture was then incubated at 37°C for 2 hours with 5%CO_2. The suspension was washed with RPMI1640 (Gibco Ltd.) thrice, centrifuging at using 200g. Then, 1 million cells were resuspended in 4 ml RPMI1640 culture medium, as described before, containing 10% foetal calf serum and 10U/ml IL-2. 3-4 days later IL-2 was topped up to 10U/ml. The culture supernatants were harvested at day 7 after the infection. Sandwich ELISA as mentioned above was used to determine the amount of p24, giving a direct indication of the amount of virus. The culture supernatants were diluted at 1:100 and 1:500 for the assay in order to obtain data in pg/ml within the reliable calibration region of the standard curve of the ELISA. The results are as follows.

Cell Treatment	Amount of p24 (pg/ml)	
	Experiment 1	Experiment 2
Untransfected cells	18,553	7,755
Cells transfected with polymerase, autopolymerase and CXCR4 ribozymal DNA vectors (3 vectors):	Not detectable	Not detectable

The above data show clearly that all HIV infectivity was inhibited by the treatment with the ribozymal vector system.

All the prior references cited herein for the purpose of referring to known materials, sequences and procedures are hereby expressly incorporated herein by reference to the extent of describing the materials, sequences and procedures referred to.

SEQUENCE LISTING FREE TEXT

Sequences deemed "artificial" for the purposes of Sequence Listing contain free text under identifier <223> as follows:

SEQ ID NO:	Free text description following "Description of Artificial Sequence"
4	DNA cassette containing T7 promoter and ribozymal DNA targeting CCR5
8	DNA cassette containing T7 promoter and ribozymal DNA targeting CXCR4
9 & 10	PCR primer containing T7 polymerase sequence
11 & 12	PCR primer containing encephalomyocarditis vurus (EMCV) 5`-UTR sequence